

DISTRIBUTION OF HUMAN PAPILLOMA VIRUS GENOTYPES IN CERVICAL CANCER TISSUES

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Abstract - Cervical cancer incidence and mortality rates in Serbia are among the highest in Europe and data on *Human papilloma virus* (HPV) type distribution are scarce. The aim of this study was to determine the prevalence of HPV types in archival specimens of cervical cancer tissues of women in the Serbian population. A total of 45 paraffin-embedded tissue samples of cervical carcinoma were used in this study. The procedure included deparaffinization of tissue samples, DNA extraction, PCR, gel electrophoresis and HPV genotyping by direct sequencing. HPV was detected in 32 samples (71%). Genotyping revealed the presence of 6 high-risk HPV types 16, 18, 33, 45, 53 and 58, where HPV type 16 was the most prevalent type (73.7%). The results of this study and further studies will provide more detailed information about HPV genotype distribution and may contribute to the formulation of national guidelines for the prevention of cervical cancer.

Key words: HPV, carcinoma, vaccination, genotyping.

INTRODUCTION

Cervical cancer is the second most frequent cancer in women worldwide in general, as well as among women between 15 and 44 years of age. Current estimates indicate that every year ~500 000 women are diagnosed with cervical cancer and ~280 000 die from the disease (WHO, 2007).

Numerous studies have demonstrated a strong and causal association between HPV and cervical cancer (Bosch et al., 2008). So far, more than 120 HPV genotypes have been identified and according to the oncogenic potential they are classified into two groups. High-risk genotypes are associated with cervical cancer while low-risk types are associated

with genital warts. All genotypes can cause an abnormal Pap test (Bernard et al., 2010; Clifford et al., 2006).

About 10% of women in the general population are estimated to harbour cervical HPV infection at a given time, and 70.1% of invasive cervical cancers in the world are attributed to HPVs 16 or 18. HPV DNA can be detected in nearly all invasive cervical lesions (Walboomers et al., 1999). Results of previous studies revealed that, on average, 93% of cervical tissues contained HPV DNA (range 75-100%) (Bosch et al., 1995). In addition, results from an updated meta-analysis showed that the overall HPV prevalence in invasive cervical cancer (ICC) was 87%, ranging from 86% to 94% by region (Smith et al, 2007).

Most commonly, HPV 16 and HPV 18 types are isolated from cervical cancer worldwide, but there are several oncogenic types of considerable importance, i.e. types 33, 45, 31, 58, 52, 35, 59 and 51 (WHO, 2007). An updated meta-analysis showed similar results of HPV type distribution for the European region (Smith et al., 2007).

Therefore, the determination of prevalence and HPV genotype distribution is very important for defining and monitoring of HPV infection and cancer prevention strategies (Bosch et al., 2008).

Cervical cancer is the leading cause of cancer mortality among women in Serbia, where the incidence of cervical cancer is among the highest in Europe. According to the data of the Cancer Registry of Serbia, from 1973-1982 the incidence was 14.7 to 18.2 per 100 000, while in the year 2002 the age-standardized incidence rate of cervical cancer was 27.2 per 100 000. Recent data from 2003-2009 showed incidence rates from 21.6 to 27.1 per 100 000 (Kesic et al., 2007; Cancer Registry of Serbia, 2011).

The data on HPV genotype distribution in cervical cancer tissues in Serbia are limited. Therefore, the aim of this study was to determine the prevalence of HPV types in archival specimens of cervical cancer tissues of women in our population.

MATERIALS AND METHODS

Tissue samples

A total of 45 formalin-fixed paraffin-embedded (FFPE) tissue samples of histologically proven cervical carcinoma diagnosed during 2009-2012 were obtained from the archives of the University Clinic of Gynecology and Obstetrics "Narodni Front", Belgrade. Sections from each tissue block were haematoxylin and eosin (H&E)-stained and assessed by the study pathologist to confirm the histological diagnosis. Depending on the tissue size, three to five 5 mm serial sections were cut from each paraffin tissue block and collected in 1.5 ml tubes for DNA extraction. To avoid cross-contamination of samples, the

microtome blade was carefully cleaned with xylene after each cut.

HPV DNA amplification and detection

The tissue sections were deparaffinised with xylene and 96% ethanol, and DNA extraction was done with a QIAamp DNA Mini Kit (QIAGEN Inc., CA, USA), according to the manufacturer's instructions.

The presence of HPV was determined by PCR method using MY09/MY11 primers for the detection of the L1 gene and GP1/GP2 primers for the detection of the E1 gene HPV DNA (Gravitt et al., 2000; Azzimonti et al., 1999). PCRs were performed in a 25 µL volume reaction mix containing Qiagen Taq PCR Master Mix-250U (QIAGEN Inc., CA, USA), 1 µmol of each primer and 5 µL of extracted DNA.

The amplification for the L1 gene was performed with the following cycling protocol: initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C and a final elongation of 20 min at 72°C (Gravitt et al., 2000). The PCR protocol for E1 gene included: initial denaturation at 85°C for 5 min, followed by 40 cycles of 1min at 94°C, 1min at 50°C, 90 s at 72°C and a final elongation of 10 min at 72°C (Azzimonti et al., 1999). A specific 450bp band for the L1 gene and 450bp for the E1 gene were detected by agarose gel electrophoresis with ethidium bromide staining.

HPV genotyping

The determination of HPV genotypes was performed by direct DNA sequencing method. HPV-positive samples were purified using the QIAGEN MinElute PCR Purification Kit (QIAGEN Inc., CA, USA). Sequencing was performed using a Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, CA, USA) with PCR primers as sequencing primers. Analysis of the HPV DNA sequences was performed by ABI Prism 310 Genetic Analyser and Sequence Analysis 5.1 software. The obtained sequences were compared with already known sequences using the BLAST program (<http://www.ncbi.nih.gov/BLAST/>)

Table 1. HPV genotype distribution in cervical carcinoma.

HPV genotype	Number of samples	% samples (binomial 95% CI)
16	14	73.84 (48.8 to 90.8)
18	1	5.26 (0.01 to 2.6)
33	1	5.26 (0.01 to 2.6)
45	1	5.26 (0.01 to 2.6)
58	1	5.26 (0.01 to 2.6)
16+53	1	5.26 (0.01 to 2.6)

in the GenBank database. The nucleotide sequence was assigned to an HPV type if it corresponded in more than 95% in 350-400bp with a known HPV genotype (Remmerbach et al., 2004; Lee et al., 2007).

RESULTS

The mean age of women with invasive cervical carcinoma included in the study was 49.31 years (range 34-75). Out of 45 cervical carcinoma tissue samples, 37 (82.2%) were squamous cell carcinomas (SCC) and 8 (17.8%) adenocarcinomas (AC).

The presence of HPV DNA was detected in 32 tissue samples (71%) using the PCR method (Fig. 1).

HPV genotype was determined in 19 of 32 positive samples. Genotyping revealed the presence of 6 high-risk HPV types: 16, 18, 33, 45, 53 and 58. The most prevalent type was HPV 16, found in 14 cases (73.84%). Other types (type 18, 33, 45, 58) were detected in one case each (5.26%). In one tissue sample (5.26%), two HPV types, 16 and 53, were identified (Table 1).

DISCUSSION

Genital HPV infection is a highly prevalent, sexually transmitted infection responsible for significant morbidity and mortality. Numerous studies have demonstrated a strong and causal association between HPV and cervical cancer (Bosch et al., 2008). The results of updated meta-analysis showed that the overall HPV prevalence in invasive cervical cancer

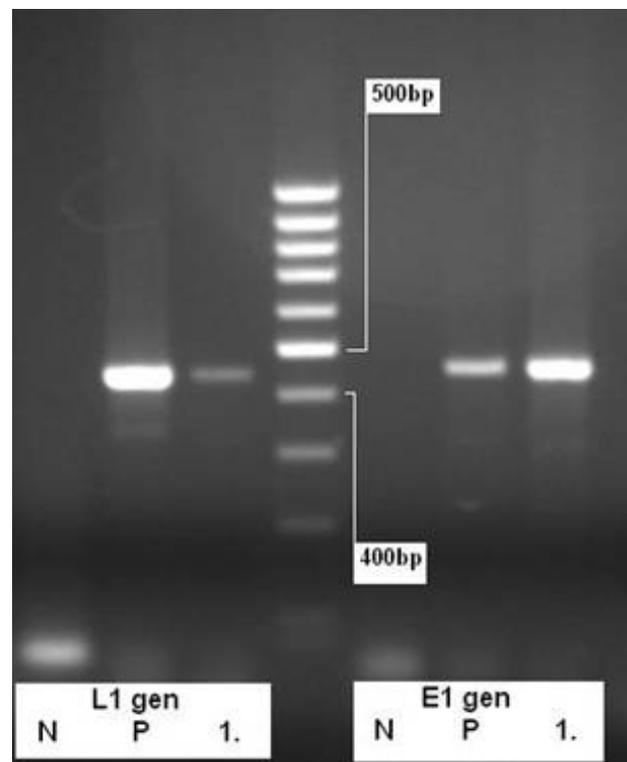


Figure 1. HPV detection by PCR (N – negative control; P – positive control; 1 – sample).

was 87%, ranging from 86% to 94% by region (Smith et al., 2007).

The overall prevalence of HPV DNA in FFPE tissue samples of invasive cervical carcinoma in our study population was 71%. This finding is similar to those reported in studies from other countries showing the prevalence of HPV in cervical carcinoma. Archival FFPE tissues are an important resource

for studies on the pathogenesis of cervical carcinoma and they have been used by many authors from different countries to investigate HPV prevalence. However, the sensitivity of PCR in FFPE tissue samples is strongly related to the size of the PCR product. Baay et al. (1996) reported that the efficacy of GP5/6, CPI/IIG and MY09/11 general primer pairs for detection of HPV DNA in FFPE carcinomas correlated with the length of the produced amplicons. The HPV positivity with GP5/6 primers (amplicon 155bp) was 61%, with CPI/IIG primers (amplicon 188bp) 57% and with MY09/11 primers (amplicon 450bp) 47%. It has been suggested that formaldehyde fixation can modify viral DNA and the amplification of a PCR product longer than 200bp is inhibited (Baay et al., 1996; Karlsen et al., 1994). The HPV positivity in FFPE tissue samples using MY09/11 and GP1/GP2 primers (amplicons 450bp) in this study was higher.

More than 120 types of HPV have been discovered and are generally classified as high-risk or low-risk types on the basis of their oncogenicity. Among the high-risk types, HPV 16 and 18 account for approximately 70% of all cases of cervical cancer. Other high-risk types such as 33, 45, 31, 58, 52, 35, 59 and 51 are detected with less frequency (WHO, 2007). Similar results of HPV type distribution in cervical carcinoma are demonstrated in the European region (Smith et al., 2007).

In our study, 6 high-risk HPV types were identified by direct DNA sequencing method in cervical carcinoma tissues (16, 18, 33, 45, 53 and 58). HPV type 16 was the most prevalent type and was found as a single infection in 14 cases (73.84%). The overall prevalence of HPV types 16 and 18 was 79.1%. This result is similar to the rates of HPV types 16 and 18 detected in FFPE tissues in Italy (67.8% and 10.7%, respectively) and in Turkey (64.7% and 9.9%, respectively), although the prevalence of HPV type 18 in these countries was higher (Rossi et al., 2012; Usubütün et al., 2009). This is not surprising, as previous studies have shown that HPV type 16 is the predominant genotype in SCC, and HPV type 18 in adenocarcinoma, so our results were compliant with histological type distribution in our samples (82.20%

SCC and 17.80% AC). In addition, these variations in HPV types 16 and 18 prevalence may be attributable to geographic differences, to the different numbers of cases included in the study, or to the use of different techniques for HPV detection, including the use of different primer pairs.

The presence of multiple HPV genotypes is less prevalent in cervical carcinoma (Molijn et al., 2005). In our study, in one cervical carcinoma tissue, two HPV types, 16 and 53, were identified.

Cervical cancer prevention programs in developed countries include HPV testing and vaccination of young women against HPV types 16 and 18. Numerous studies showed that the vaccines are highly effective in preventing the development of cervical precancerous lesions associated with HPV type 16 or 18 (Castellsague et al., 2009; Lu et al., 2011).

Therefore, the results of this study indicate that HPV types 16 and 18 are important risk factors for cervical cancer in Serbia and that at least 79% of cervical cancer cases in this country are potentially preventable by currently available prophylactic HPV vaccines. Furthermore, the results of this study and further studies will provide more detailed information about HPV genotype distribution in cervical cancer tissues of women in our population and may contribute in the formulation of national guidelines for the prevention of cervical cancer.

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